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14. ABSTRACT

Although the importance of microenvironment in prostate cancer is widely recognized, the molecular and cellular processes leading from genetic changes in the prostatic epithelium to the establishment of a tumorigenic microenvironment for prostate cancer is unclear in most contexts. With our finding of NFATc1 being an oncogene and has a potential role in prostate cancer, we proposed to study two main areas (divided into 3 specific aims). **First**, the detailed study of the tumorigenic microenvironment and the correlation between NFATc1 and prostate cancer status in humans will help facilitate the development of clinically useful biomarkers for both diagnostic and prognostic purposes. Many of the factors we are targeting in the prostate cancer microenvironment are secreted factors that may be present in serum and/or urine at measurable levels, making them suitable for the development of non-invasive clinical tests. **Second**, the illustration of the main cellular and molecular components in the tumorigenic microenvironment provides new druggable targets aimed at reversing the effects of the alterations in the microenvironment.

15. SUBJECT TERMS
prostate cancer, microenvironment, oncogene, senescense, NFAT, cytokines,

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TABLE OF CONTENTS

1. INTRODUCTION:

Based on our preliminary data revealing a role of NFAT activation in prostate cancer (prostate cancer), we hypothesize that NFATc1 promotes prostate cancer by regulating oncogenic proteins in the prostatic epithelium and by non-cell autonomous effects on other cells through secreted factors. These factors initiate a cascade of reciprocal events between the prostatic epithelium and stroma, leading to the creation of an inflammatory and pro-mitogenic microenvironment for prostate cancer development. Besides testing this hypothesis and to examine the interactions between NFATc1 and known oncogenic factors/tumor suppressors, we will further reveal the key players in the prostate cancer microenvironment and to explore the potential of NFATc1 as a novel biomarker for prostate cancer diagnosis/prognosis. We will take advantage of the cellular precision, genetic manipulability, and on-off inducibility of our murine model to further study the tumorigenic processes initiated by NFATc1 activation in the prostate (Aim 1) as well as the key molecular and cellular components in the NFATc1-induced tumorigenic microenvironment (Aim 2). In Aim 3, we will study the involvement of NFATc1 activation in human prostate cancer and the oncogenic effects of NFATc1 in human prostate cancer cells.

2. KEYWORDS:

prostate cancer, microenvironment, oncogene, senescence, NFAT, cytokines,

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

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Partnering PI: Zongbing You

Specific Aims and tasks (specified in proposal)	**Timeline (**Months)	**Site 1** Washington University	**Site 2** Tulane University	**Actual completion date**
Specific Aim 1: Investigate the tumorigenic processes initiated by NFATc1 activation in prostate				
Major Task 1: Investigate the tumorigenic processes initiated by NFATc1 activation in prostate				
Subtask 1: Investigate if NFATc1-induced prostate cancer progresses into metastatic prostate cancer	1-24	Drs. Chen, Manda, Tripathi, Ding, Andriole	Dr. You	Partially completed (Please see details immediately following this table)
Subtask 2: Investigate if NFATc1 promotes the progression of hormone-naïve prostate cancer into castration-resistant prostate cancer	1-18	Drs. Chen, Manda, Tripathi, Ding, Andriole	Dr. You	Completed (A portion of these results was described in the previous report and the Oncogene manuscript)
Subtask 3: Investigate if termination of NFATc1 activation halts prostate cancer progression	1-8	Drs. Chen, Manda, Tripathi, Ding,	Dr. You	Completed. 04/2015 (A portion of these results was

		Andriole		described in the previous report and the Oncogene manuscript)
Milestone(s) Achieved: Determine the in vivo role of NFATc1 activation in prostate cancer initiation and progression	24	Drs. Chen, Manda, Tripathi, Ding, Andriole	Dr. You	Some of the results have been included in a manuscript.
Major Task 2: Study the potential synergy between NFAT signaling and Pten/PI3K/Akt in prostate cancer				
Subtask 1: Study if NFATc1 activation overcomes *Pten* inactivation-induced senescence.	1-6	Drs. Chen Dr. Chen, Manda, Tripathi, Ding, Maher (90 mice will be used)	Dr. You	Completed 05/2015 (A portion of these results was described in the previous report and the Oncogene manuscript)
Subtask 2: investigate if NFATc1 activation promotes prostate cancer bone metastasis in Pten mutants	1-24	Drs. Chen Dr. Chen, Manda, Tripathi, Ding, Maher (90 mice will be used)	Dr. You	Mostly completed (Please see details in sections following this table)
Milestone(s) Achieved: Determine the interactions between NFATc1 and Pten in prostate cancer	24	Drs. Chen, Manda, Tripathi, Ding, Maher	Dr. You	Some of the results have been included in a paper.
Specific Aim 2: Reveal the critical components in NFATc1-induced tumorigenic microenvironment and evaluate the importance of SPP1, a potential NFATc1 target, in NFATc1-induced prostate cancer				
Major Task 3: Study the NFATc1-induced tumorigenic microenvironment and the role of SPP1 in prostate cancer				

Subtask 1: Further analyze the cellular and molecular components in the prostate cancer microenvironment	10-34	Drs. Chen, Tripathi, Manda, Ding (72 mice will be used)	Dr. You	Ongoing (Please see details in sections following this table)
Subtask 2: Study the role of SPP1, an NFATc1 target, in NFATc1-induced prostate cancer	14-36	Drs. Chen, Tripathi, Manda, Ding (300 mice will be used)	Dr. You	Ongoing (Please see details in sections following this table)
Milestone(s) Achieved: Provide molecular details to the NFATc1-induced tumorigenic microenvironment and determine the connections between NFATc1 and SPP1	36	Drs. Chen, Tripathi, Manda, Ding	Dr. You	Ongoing (Please see details in sections following this table)
Specific Aim 3: Investigate NFAT signaling in human prostate cancer specimens and human prostate cancer cell lines				
Major Task 4: Determine if there is a direct connection between NFATc1 expression and human prostate cancer pathogenesis				
Subtask 1: Determine if there is a connection between NFATc1 expression and human prostate cancer grade/stage	1-36	Drs. Chen, Manda, Tripathi, Ruzinova, Hsi, Ding, Maher, Andriole (275 human prostate cancer specimens)		Ongoing (Please see details in sections following this table)
Subtask 2: Investigate the oncogenic effects of NFAT signaling in human prostate cancer cell lines	16-32	Drs. Chen, Manda, Tripathi, Ding, Maher	Dr. You	Partially complete (Please see details in sections following this table.)
Milestone(s) Achieved: Determine if NFATc1 can be a biomarker for	36	Drs. Chen, Manda,		This part of the study is still

prostate cancer progression in human and further understand the effect of NFATc1 activation in human prostate cancer cells		Tripathi, Ruzinova, Hsi, Ding, Maher, Andriole		Ongoing (Please see details in sections following this table)

What was accomplished under these goals?

1) Major activities

Major Task 1: Investigate the tumorigenic processes initiated by NFATc1 activation in prostate

We have completed the study of the effects of antigen deprivation in mice with NFAT activation in the prostate.

We have completed the study if termination of NFATc1 activation halts prostate cancer progression in the NFAATc1-induced murine prostate cancer model.

We have so far not found any evidence of metastatsis of the NFATc1-induced prostate cancer in mice.

Major Task 2: Study the potential synergy between NFAT signaling and Pten/PI3K/Akt in prostate cancer

We have demonstrated the synergy between NFAT signaling and Pten/PI3K/Akt signaling in prostate cancer.

We have shown that NFATc1 has anti-senescence effects and such effects overcome Pten inactivation-associated cellular senescence.

Major Task 3: Study the NFATc1-induced tumorigenic microenvironment and the role of SPP1 in prostate cancer

We have further analyzed the cellular and molecular components in the prostate cancer microenvironment. More work is ongoing in this area.

We have produced some mice necessary for the study of the role of SPP1, an NFATc1 target, in NFATc1-induced prostate cancer. More work is ongoing in this area.

Major Task 4: Determine if there is a direct connection between NFATc1 expression and human prostate cancer pathogenesis

We have conducted an initial study of a potential connection between NFATc1 expression and human prostate cancer progression. More work is ongoing in this area.

We have investigated the oncogenic effects of NFAT signaling in human prostate cancer cell lines. Our ongoing studies will continue to provide useful information about the molecular and cellular mechanism of NFATc1driven oncogenesis in the prostate and other organs.

2) Specific objectives

Our main objectives are:
Aim 1: Investigate the tumorigenic processes initiated by NFATc1 activation in the prostate.

Aim 2: Reveal the critical components in NFATc1-induced tumorigenic microenvironment and evaluate the importance of SPP1, a potential NFATc1 target, in NFATc1-induced prostate cancer.

Aim 3: Explore the potential of NFATc1 as a novel diagnostic/prognostic marker and study the role of NFATc1 in human prostate cancer cell lines.

3) Significant results

Major Task 1: Investigate the tumorigenic processes initiated by NFATc1 activation in prostate

1.1: Study the effects of castration in mice with NFAT activation in prostate:

We have found that NFAT signaling can overcome castration to drive prostate cancer progression.

We are not presenting the actual data here since part of these data was presented in our Oncogene paper[1] and in the progress report last year. Here is a brief summary of what has been done in this area.

Since androgens are critical both for development and function of the prostate gland and for the survival and proliferation of the epithelial cells,[2] androgen deprivation has been a key therapeutic strategy in combating prostate cancer progression. In order to determine if NFATc1-induced prostate cancer would respond to hormone deprivation therapy, such as castration, we analyzed prostates from 18-week-old mutant mice with NFATc1 activation since weaning and were either castrated (by surgically removing both testicles) or mock-castrated at 14 weeks of age. We thus only compared the results between castrated and mock-operated mice at the end point (18 weeks of age). Prostate cancer samples from castrated and mock-castrated mutants are similar in tumor size and histopathological features, suggesting that NFAT signaling can overcome castration to drive prostate cancer progression.

1.2: Study if termination of NFATc1 activation halts prostate cancer progression in the murine prostate cancer model

We have found that tumor progression and survival depend on activation of NFATc1*.*
We are not presenting the actual data here since part of these data was presented in our Oncogene paper[1] and in the progress report last year. Here is a brief summary of what has been done in this area.

We used allografts of NFATc1-induced prostate cancer onto nude mice to study if the progression and survival of the NFATc1-induced prostate cancer cells continue to depend on NFATc1 activation. We have first generated multiple prostate cancer cell lines from the NFATc1-induced prostate cancer by dissociating the dissected prostate cancer from mutant mice into single cells and cultured them with doxycycline (Dox). These tumor cells were then injected to the rear flanks of the nude mice. Recipient mice treated with Dox showed growth of tumor as early as 4 weeks after the injection. Tumor growth was not observed, however, in the untreated (without NFATc1 activation) recipient mice. Existing tumors started to shrink within days after Dox withdrawal. This trend was reversed when NFATc1 activation was restored with Dox treatment. These results indicate a continuous dependency of the prostate cancer on NFATc1 activation, similar to that seen in cases of oncogene addiction. Histopathological analyses of tumors revealed that these allografts contained carcinoma with a more solid growth pattern but showed cytological features similar to those seen in original tumors. We are not presenting the actual data here since part of these data was presented in our Oncogene paper[1] and in the progress report last year.

1:3: Investigate if NFATc1-induced prostate cancer progresses into metastatic prostate cancer

Several studies have shown that NFATc1 is associated with the progression of multiple types of cancers.[1, 3-18] To determine whether NFATc1-induced prostate cancer in mice can metastasize, we sacrificed and analyzed the lymph nodes, lung, liver and bones (mainly tibia, femur and spinal vertebrae) of mice with induced NFATc1 activation in the prostatic epithelium for various length of time, from 14 weeks to about 6 months. Due to the overall tumor burden and other confounding problems in these mice, only 2 mice lived past 6 months after NFATc1 activation. Figure 1 has representative data from one of such mutant mice.

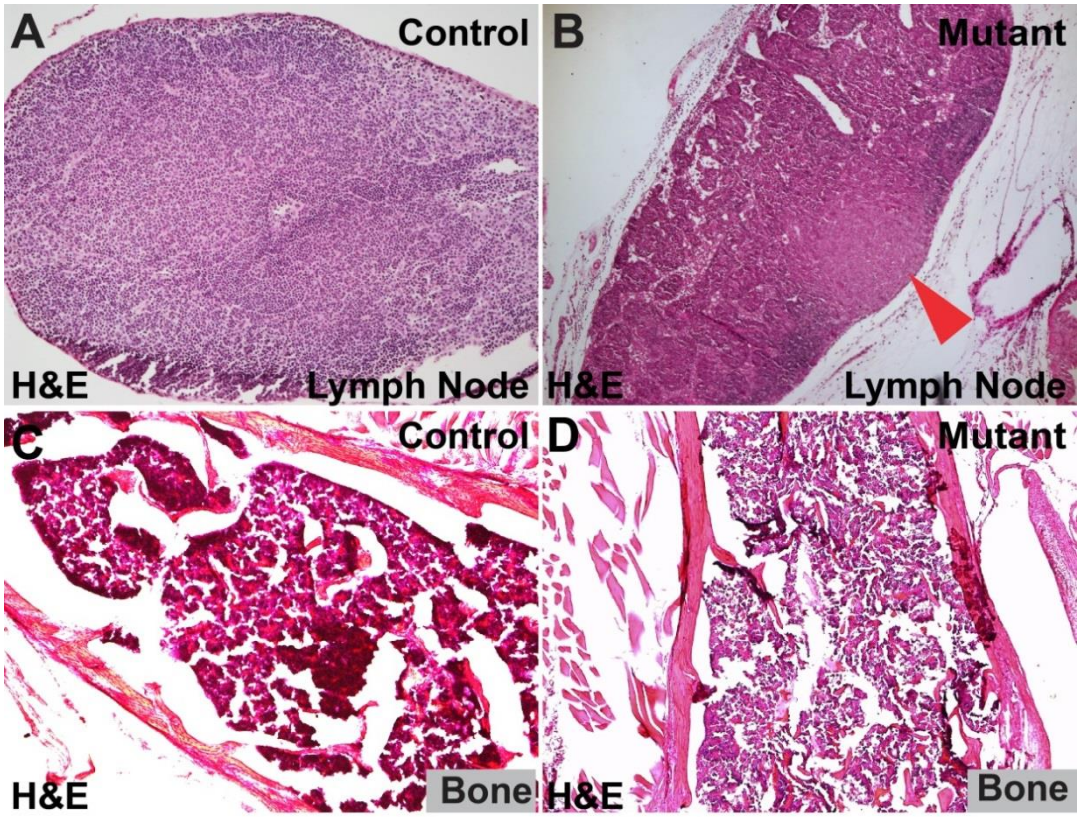

Figure 1. No clear evidence of metastasis in mice with NFATc1-induced prostate cancer has been found yet.

We have found some unexplained and unusual cells and cell masses in the lymph nodes occasionally (Figure 1A-B), but we have not found any clear evidence of metastasis in the bones (Figure 1C-D) or other organs (data not shown). It is still unclear what type of cells made up the unusual mass in some of the mutant lymph nodes. Morphologically, they do not appear to resemble prostate cancer cells. Their identity and significance are still under investigation. It is possible that these are results of immune responses rather than metastasis. Although we are still looking at more samples with longer duration of NFATc1 activation, we have shifted our attention to mutants with both NFATc1 activation

and PTEN inactivation to test the hypothesis that additional genetic mutations may promote the NFATc1-induced prostate cancer to progress further, including metastasis. Please see section 2.3.

Major Task 2: Study the potential synergy between NFAT signaling and Pten/PI3K/Akt in prostate cancer

2.1: Study the synergy between NFAT signaling and Pten/PI3K/Akt signaling in prostate cancer.

We found that NFATc1 activation synergizes with the PI3K-AKT pathway to promote prostate cancer progression.

We are not presenting the actual data here since part of these data was presented in our Oncogene paper[1] and in the progress report last year. Here is a brief summary of what has been done in this area.

Tumor suppressor PTEN is frequently mutated s in prostate cancer.[19, 20] To understand if and how the NFAT and PI3K-AKT pathways interact in prostate cancer, we generated mice with both PTEN deficiency and NFATc1 activation in prostatic epithelia. At 10 weeks of age, most mice with only PTEN deficiency in the prostate epithelium (*PCre (*Probasin-Cre*)/+;Pten$^{fl/fl}$*) showed enlarged anterior prostates, whereas control and mice with only NFATc1 activation (*PCre/+;RT(ROSA-rtTA)/+;TN(tetO-NFATc1Nuc)/+*) starting from P21 in prostatic epithelium had no visible tumors. Interestingly, all double mutants (*PCre/+;RT/+;TN/+;Pten$^{fl/fl}$*) with both PTEN deficiency and NFATc1 activation developed significantly larger tumors in all prostate lobes when compared to mice of the same age with either PTEN deficiency or NFATc1 activation alone . Histopathological analyses revealed that *PTEN* null mice and mice with NFATc1 activation alone had PIN at this time, whereas double mutants already had poorly differentiated prostatic adenocarcinoma. These findings revealed that NFATc1 activation synergizes with PTEN-AKT pathway, especially the activation of AKT, for prostate cancer initiation and progression.

2.2: Study if NFATc1 has anti-senescence effects and if such effects overcome Pten inactivation-associated cellular senescence.

We found that NFATc1 activation overcomes PTEN-loss-induced cellular senescence through down regulation of cell cycle inhibitors.

We are not presenting the actual data here since part of these data was presented in our Oncogene paper[1] and in the progress report last year. Here is a brief summary of what has been done in this area.

Senescence plays a tumor-suppressive role in PTEN-deficient cells, explaining the long tumor latency in murine models with PTEN-deficient prostate.[20] The earlier onset and faster progression of prostate cancer in PTEN-NFAT double mutants suggest that NFATc1 activation may allow the tumor cells to avoid the PTEN-loss-induced cellular senescence, resulting in accelerated tumor growth. Our study also revealed that there was a marked decrease in the expression of the senescence marker p21 in *PCre/+;RT/+;TN/+;Pten*$^{fl/fl}$ samples when compared with the *PCre/+;Pten*$^{fl/fl}$ mice. [21, 22] To confirm that NFATc1 activation overcomes PTEN-loss-induced cellular senescence, we stained for senescence-associated β-galactosidase (SA-β-gal) activity in the prostates. Control and *PCre/+;RT/+;TN/+* prostates showed very few senescent cells, 1% *and* 6.66±0.5%, respectively. In contrast, 65.6±8.7% cells within the *PCre/+;Pten*$^{fl/fl}$ prostates were SA-β-gal^{+}. Such SA-β-gal^{+} cells in the *PCre/+;RT/+;TN/+;Pten*$^{fl/fl}$ prostates were dramatically reduced to 5.8±1.3%, supporting the hypotheses that NFATc1 overcomes Pten-induced cellular senescence by down regulating cell cycle inhibitors.

2:3: Investigate if NFATc1 activation promotes prostate cancer bone metastasis in Pten mutants

In our earlier aims, we have shown that NFATc1 activation interacts synergistically with the PI3K-PTEN-AKT pathway in driving the progression of the prostate cancer in mice by overcoming PTEN loss-induced senescence. We next tried to determine if NFATc1-induced prostate cancers with the additional PTEN inactivation can metastasize to other organs. Since prostate cancers in the double mutants grow very aggressively and cause severe morbidity and death in the mice bearing these cancers, we have so far only managed to analyze double mutant mice with NFATc1 activation for about 7 weeks. We harvested and analyzed the lymph nodes, lung, liver and bones (mainly tibia, femur and spinal vertebrae) from the mutant and control mice treated with Dox for up to 7 weeks. We used H&E and immunofluorescence staining to determine if any metastases occurred. Briefly, we have not found clear evidence of metastasis of the NFATc1-induced prostate cancer in bone and other organs in these relatively young mice (Figure 2). There were occasional unusual findings of NFATc1+ cells in the mutants, such as in the lung section in Figure 2B. However, since these cells are not E-Cad positive, we do not consider them to be metastatic prostate cancer cells. The investigation of whether or not NFATc1-induced prostate cancers can metastasize is ongoing and we are exploring different approaches to address this issue as the tumor burden and comorbidities making studying double mutants carrying the NFATc1-induced prostate cancer for longer periods a challenge. One of the approaches we are exploring is to study the orthotopic allografts tumors in nude mice without some of the comorbidities.

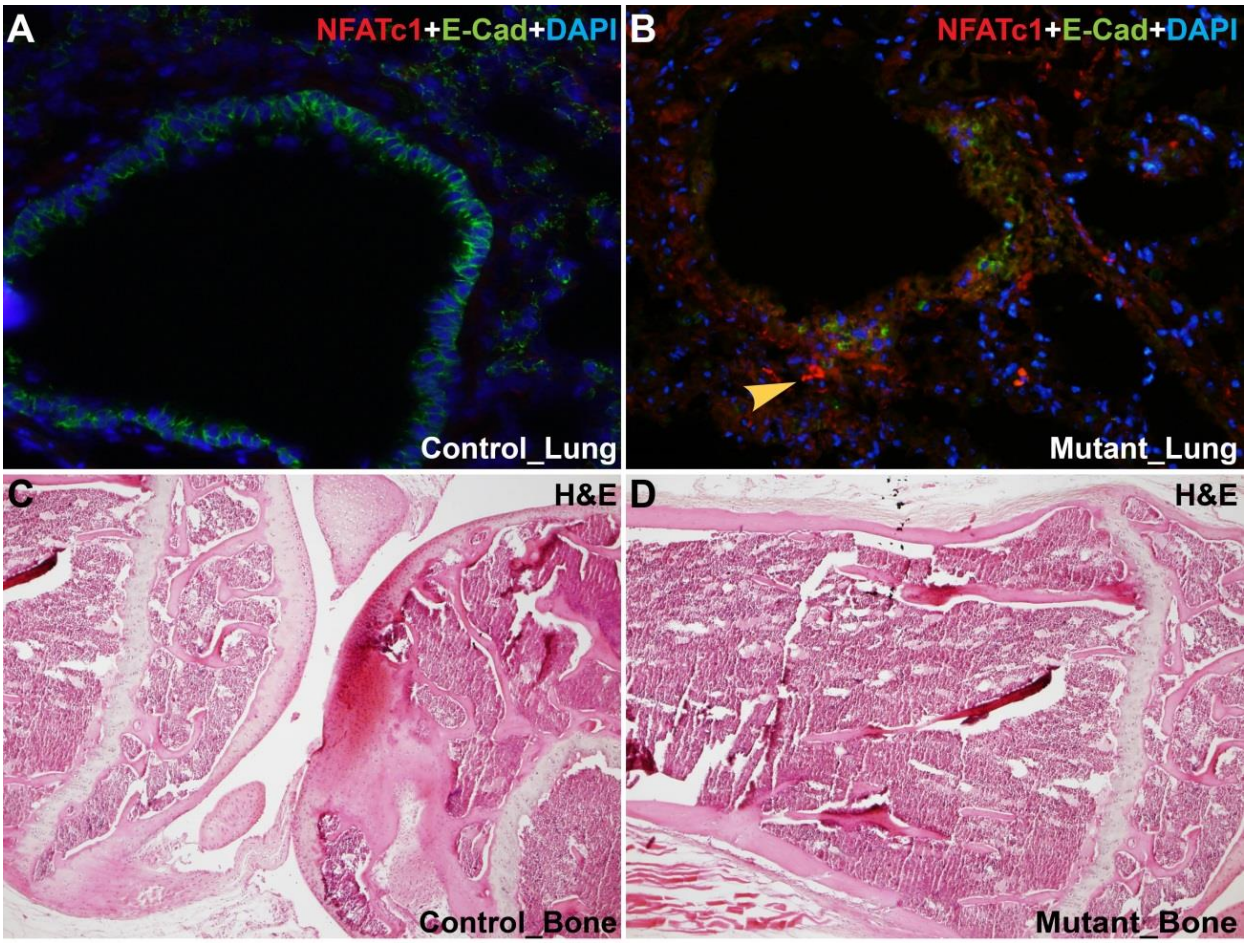

Figure 2: No evidence of metastasis has been found in mice carrying NFATc1-induced prostate cancer with additional PTEN deficiency for a relatively short period of time. *Unexplained appearance of NFATc1+ cells in lung tissue in some mutants was noted (B). But these are not considered signs of metastasis since there was not E-Cad+ NFATc1+ cells found.*

Major Task 3: Study the NFATc1-induced tumorigenic microenvironment and the role of SPP1 in prostate cancer

3:1. We further analyzed the cellular and molecular components of the prostate cancer microenvironment

Molecular Components:

We hypothesize that, in addition to cell autonomous effects, NFATc1-induced transcriptional changes in the prostatic epithelium directly and indirectly lead to the production of secreted factors, including SPP1 (a reported NFAT target and a key prognostic marker for prostate cancer that is upregulated in the NFATc1-induced prostate cancer), capable of establishing a permissive/conducive microenvironment for prostate cancer development. To test this hypothesis, we have investigated changes in some key cellular and molecular components in this tumorigenic microenvironment as a result of NFATc1 activation in the prostatic epithelium. After harvesting the prostate samples from control and mutant mice (capable of NFATc1 activation in the prostatic epithelium under Dox treatment), total RNA was isolated by using the Trizol reagent (Life Technologies) and purified with an RNeasy Mini Kit (Qiagen). cDNA was prepared from RNA using the Invitrogen ThermoScript™ RT-PCR System (Life Technologies). We have found upregulation of a range of cytokines and secreted factors implicated in cancer progression. Some of these are shown in Figure 3, including SAA1, SAA3, SPP1, CCL3, CCL4, S100A8, IL6, IL1α, IL1β, and others at transcriptional levels.

Direct upregulation of cytokines and secreted factors by NFATc1 activation

The above data as well as some of the immunofluorescence staining results on tissue section presented in our previous report and the Oncogene paper have clearly shown the increased presence of multitudes of cytokines and other secreted factors in the prostate cancer microenvironment.

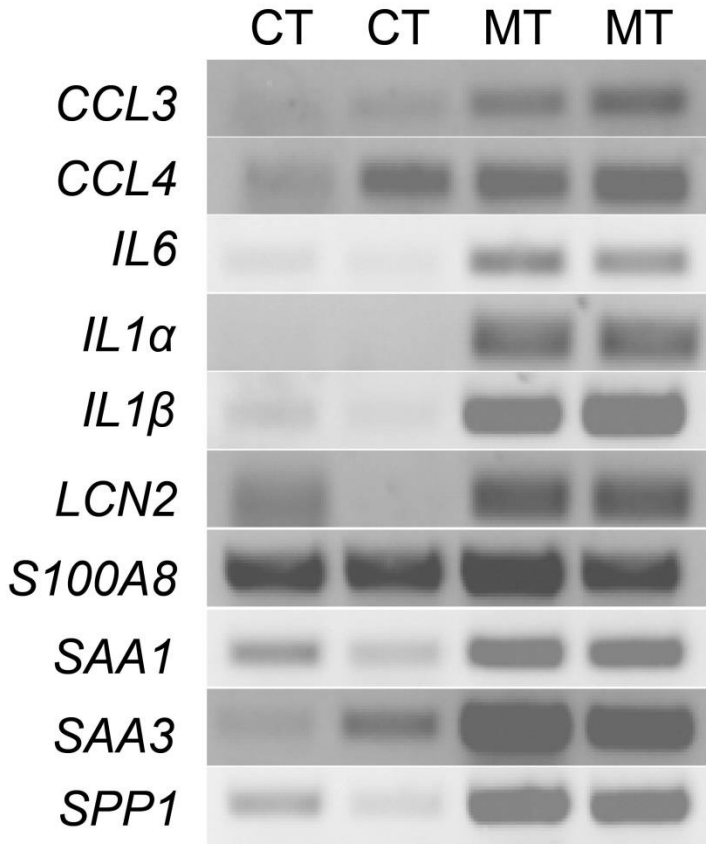

Figure 3: Molecular changes in the tumor microenvironment. *A number of secretory factors known to play a role in prostate cancer progression like, SPP1, IL6, and IL1, along with several other genes were evaluated for transcriptional changes with RT-PCR using RNA from prostate of control (CT) and mutant (MT) mice treated with Dox for ~14 weeks.*

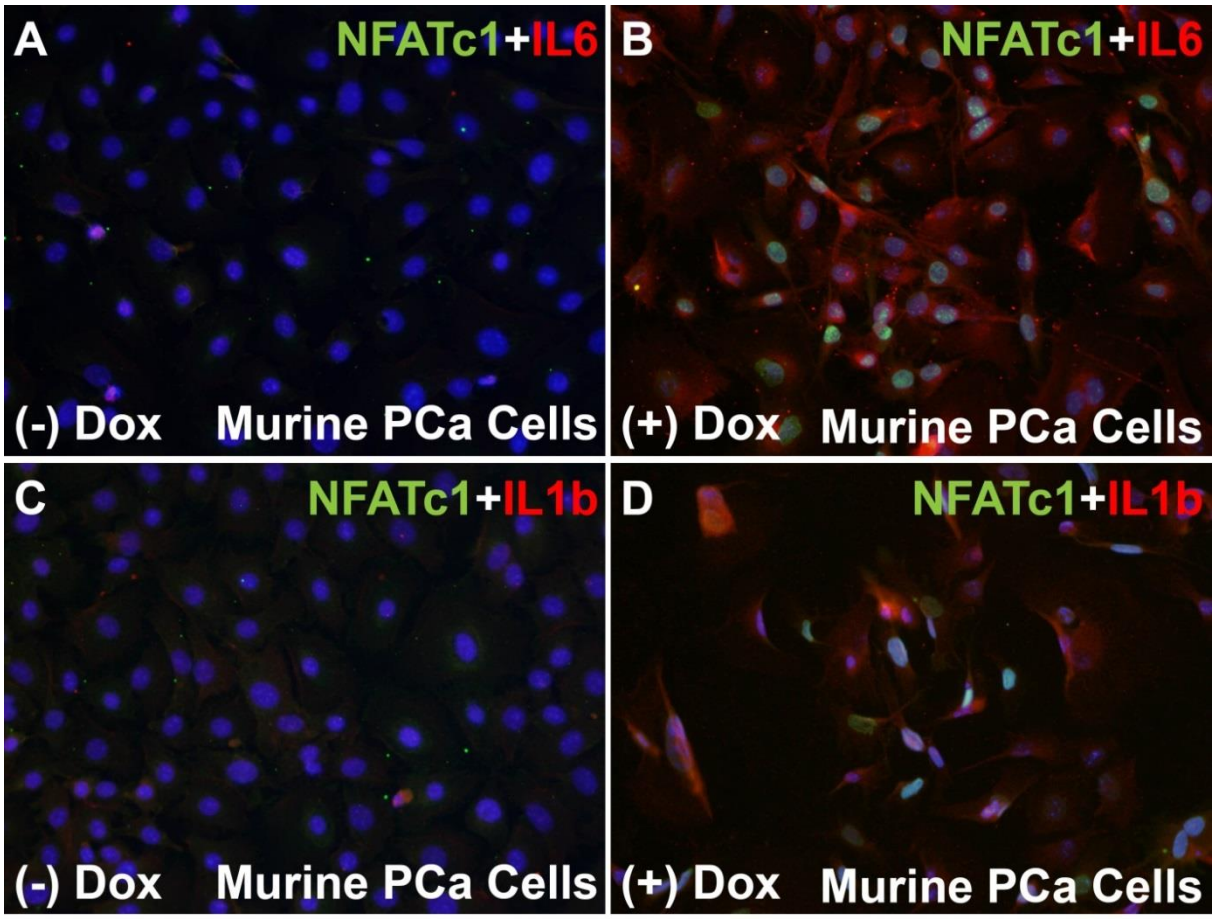

Figure 4: Increased expression of IL6 and IL1β in murine prostate cancer cells as a direct result of NFATc1 activation.

To clarify if the upregulation of these factors is a result of increased production of them in cells with NFATc1 activation or as secondary responses, we studied the expression changes of selected factors in isolated cancer cells, outside of the in vivo environment, to see if NFATc1 activation directly causes these cells to produce more cytokines and other secreted factors. For primary tumor cell culture, prostate from 14-week-old mutants (*PCre/+;RT/+;TetO-NFATc1*^{Nuc}) were harvested and cut into small pieces of <1mm and cultured in Dulbecco's Modified Eagle's Medium-F12 (10% FBS, 5%

penicillin/streptomycin and 2µg/ml Dox). Cells grew out of the tumor tissue chunks were fed with fresh media every 2-3 days, and sub-cultured before confluence. Cultured cells were further purified by clonal selection. Colonies exhibiting epithelial cell morphology were isolated by using clonal rings, trypsinized and sub-cultured. One of the clonal lines was further expanded and used for cell culture studies. Cultured cells were tested for nuclear NFATc1 expression in the presence of Dox.

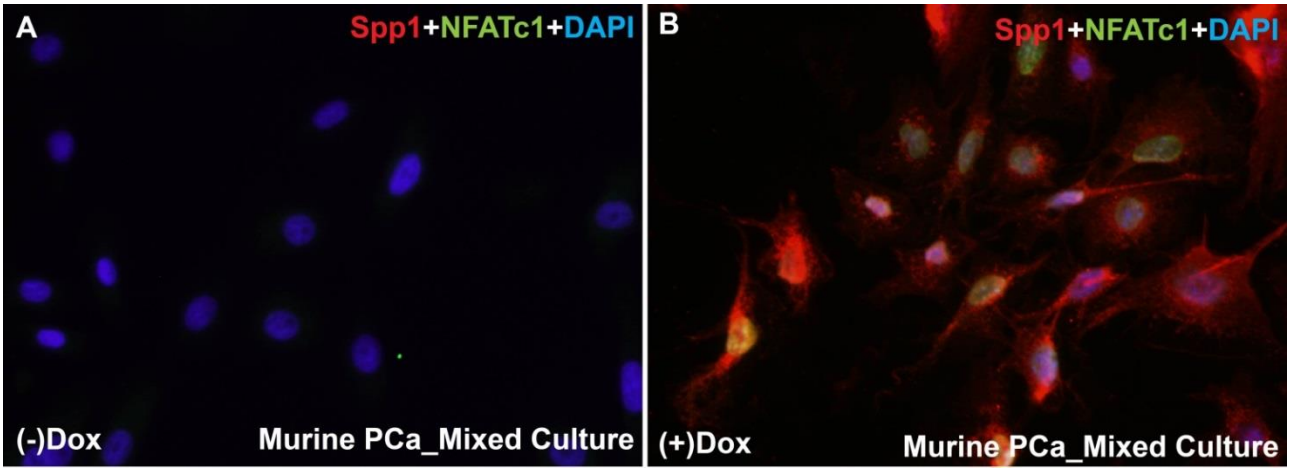

Figure 5: NFATc1 drives SPP1 expression in murine prostate cancer cells. *The murine prostate cancer cells we generated from NFATc1-induced prostate cancer do not express SPP1 without Dox treatment. When treated with Dox, these cells showed NFAT activation and greatly increased Spp1 expression (A-B).*

Multiple recent studies have collectively indicated that SPP1 is one of the four key signatures genes correlated with prostate cancer progression and prognosis.[23] Thus, we have also examined if the production of SPP1 in these murine tumor cells are NFATc1 dependent. As shown in Figure 5, SPP1 upregulation is seen in Dox treated cells, but not in the non-treated cells, demonstrating again a strong correlation between NFATc1 activation and the increased production of SPP1. This is consistent with some earlier reports that NFATc1 may regulate SPP1.[24]

We have demonstrated in these experiments that the increased expression of multiple cytokines and other secreted factors are a direct result of NFATc1 upregulation in the prostatic epithelial cells. The upregulation of these factors are key events in the further establishment of a proinflammatory and promitogenic microenvironment that will affect cellular behavior.

Cellular components of tumorigenic microenvironment

In addition to delineating the key molecular components of this tumorigenic microenvironment, we have also revealed some the key cellular components of the inflammatory environment by studying local and infiltrating cells. We found extensive infiltration of CD3+ T cells (Figure 6A-B) in the NFATc1-induced prostate cancers. Along with T cells, we also found significant number of F4/F80^{+} macrophages (Figure 6C-F) in prostate cancer but not the control samples after 3 months of NFATc1 activation. Immune cell infiltration occurs after 2 days, since at that time, no significant increase of macrophage (Figure 6C-D) or T cells (not shown) has occurred. The exact timing or the infiltration and the potential presence of other cell types will be further studied.

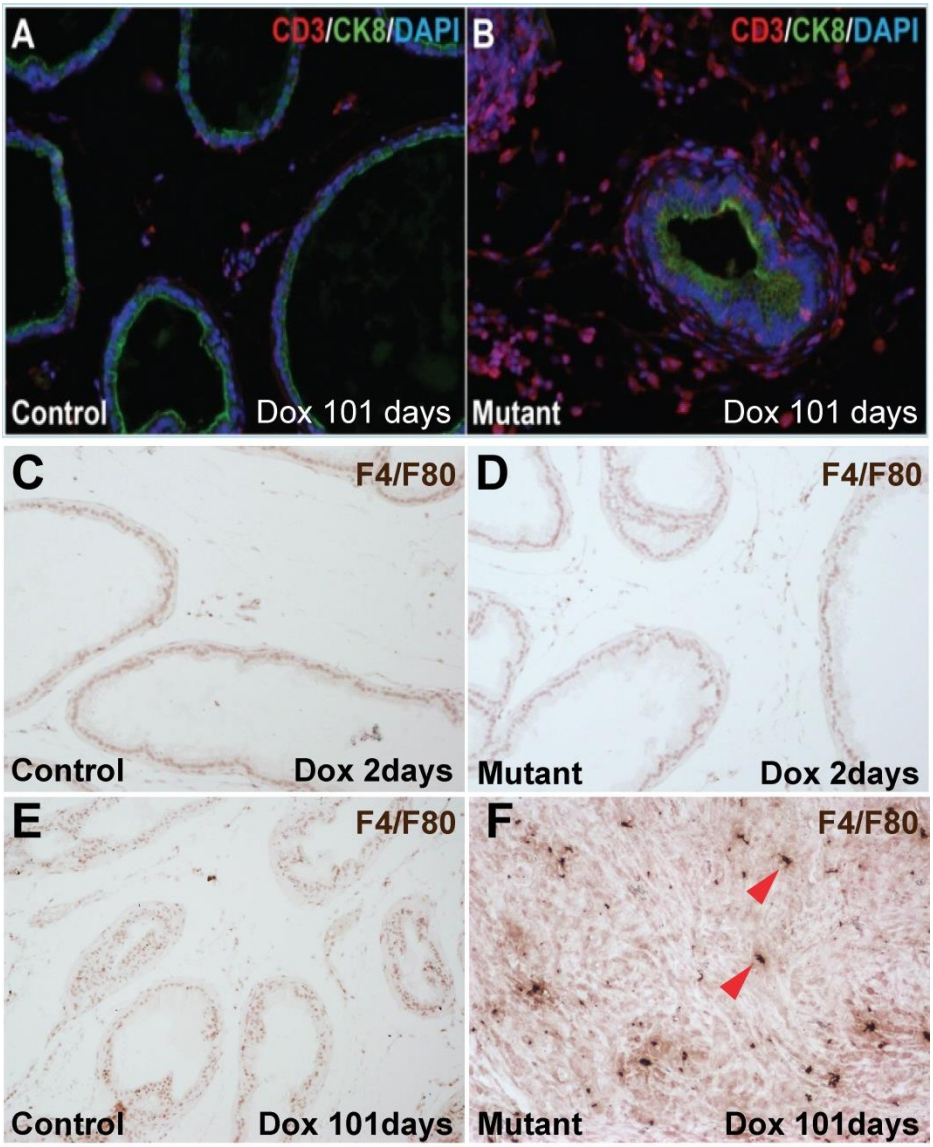

Figure 6: Infiltration of immune cells is an important process in the establishment of a pro-inflammatory and pro-mitogenic microenvironment. *Extensive infiltration of CD3+ T cells in prostate cancers in the Dox-treated mutants when compared to control prostate (A-B). Significant number of F4/F80+ macrophages was present in prostate cancers in mutants treated with Dox for 14 weeks but not in the controls. This appears to be a late event since prostates from both controls and mutants treated with Dox for 2 days did not show any macrophage infiltration (C-F).*

Wnt signaling is thought to be important in prostate cancer, in part because proteins such as β-catenin can also affect androgen receptor signaling.[25-31] Besides being part of a cell adhesion complex with E-cadherin, β-catenin is also an essential component in transducing Wnt signaling on the cell membrane to the transcriptional responses in the nucleus through the canonical Wnt signaling pathway.

A number of studies have reported the altered expression and/or localization of β-catenin as a biomarker in prostate cancer. NFAT can activate COX2, c-Myc, Wnt, Frizzled, SFRP2 and others to cause increased cell migration, metastasis, and angiogenesis. Wnt activates its canonical β-catenin-TCF pathway for signal transduction and transcription in the nucleus. Non-canonical Wnt pathway can also activate NFATc1 pathway, causing expression of several Wnt responsive genes. We wanted to determine if NFATc1-induced prostate cancer has an alteration in β-catenin expression. We found upregulation of β-catenin in NFATc1-induced prostate cancers when compared to control (**Figure 7**). The exact effects of β-catenin upregulation in the progression of NFATc1-induced prostate cancer are still being investigated.

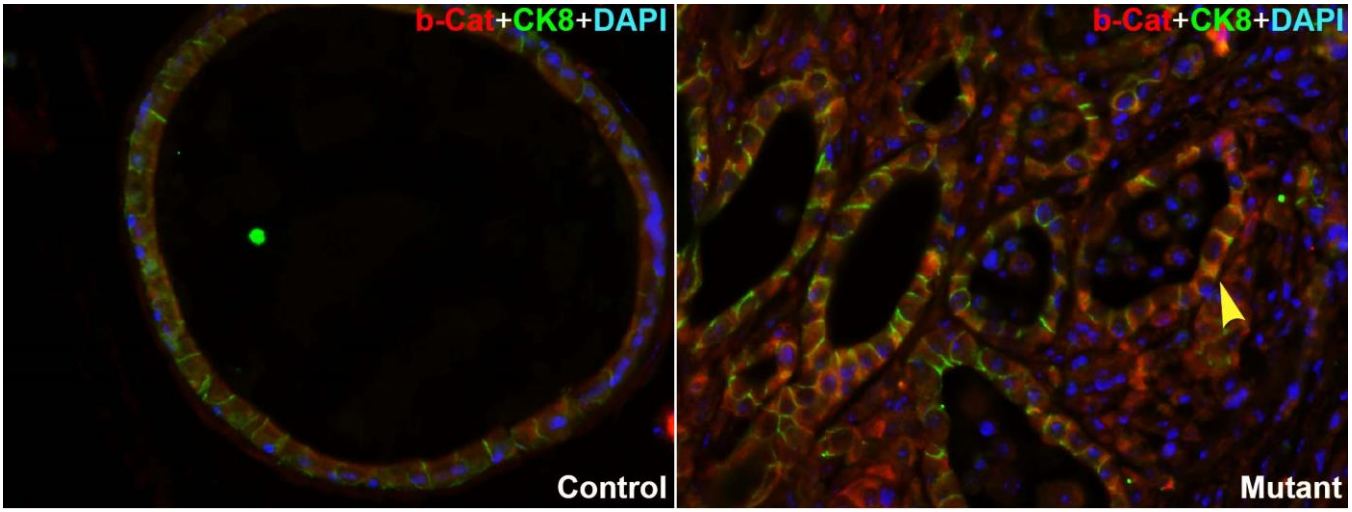

Figure 7: NFATc1 activation upregulates β-catenin, an important mediator of canonical Wnt signaling.

CK8+ cells in normal murine prostates do not show any β-catenin expression whereas murine NFATc1-induced prostate cancers showed upregulated β-catenin expression.

We are also generating mice with NFATc1 activation in the prostatic stroma (by using the TBx18Cre transgene we made) instead of prostatic epithelium in order to test if changing of the environment is sufficient for prostate cancer development.

3.2 Study the role of SPP1, an NFATc1 target, in NFATc1-induced prostate cancer.

SPP1 has been indicated as a key biomarker for prostate cancer.[23] Previous experiments have also shown that SPP1 can be activated by NFAT signaling and serves as a major downstream factor of the NFAT effects in smooth muscle cells.[32] By using the cultured cell line we generated from the NFATc1-induced murine prostate cancer, we showed that SPP1 is upregulated in the NFATc1 positive cells (**Figure 8**).

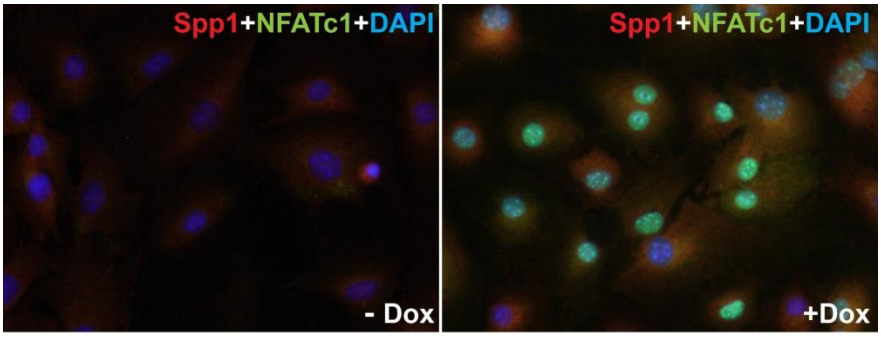

Figure 8: Removal of Dox diminishes Spp1 expression in murine prostate cancer cells.

We have generated some of the mice necessary to evaluate the importance of SPP1 in the oncogenic effects of NFATc1 in prostate cancer through studying the effects of inactivating SPP1 in mice with NFATc1 activation in prostatic epithelium and the *in vivo* effects of upregulation of *SPP1* in the prostate for prostate cancer development, with or without NFATc1 activation in the prostatic epithelium. We have generated the tetO-SPP1 transgenic mice (Tsp/+) mice. Tsp/+ mice are viable and fertile and live normal without any obvious defects. By combining the PCre (Probasin-Cre) transgene, the ROSA-rtTA allele, and the Tsp transgene, we have generated some mutants capable of Dox-mediated upregulating SPP1 in prostatic epithelium (PCre/+; ROSA-rtTA, Tsp/+) and controls that cannot upregulate SPP1

even in the presence of Dox. The expression of SPP1 in these was evaluated as revealed by immunofluorescence (**Figure 9**). Dox-treated mutant mice showed elevated expression of SPP1 in and near the CK8+ prostatic epithelium whereas control mice did not show any significant signals.

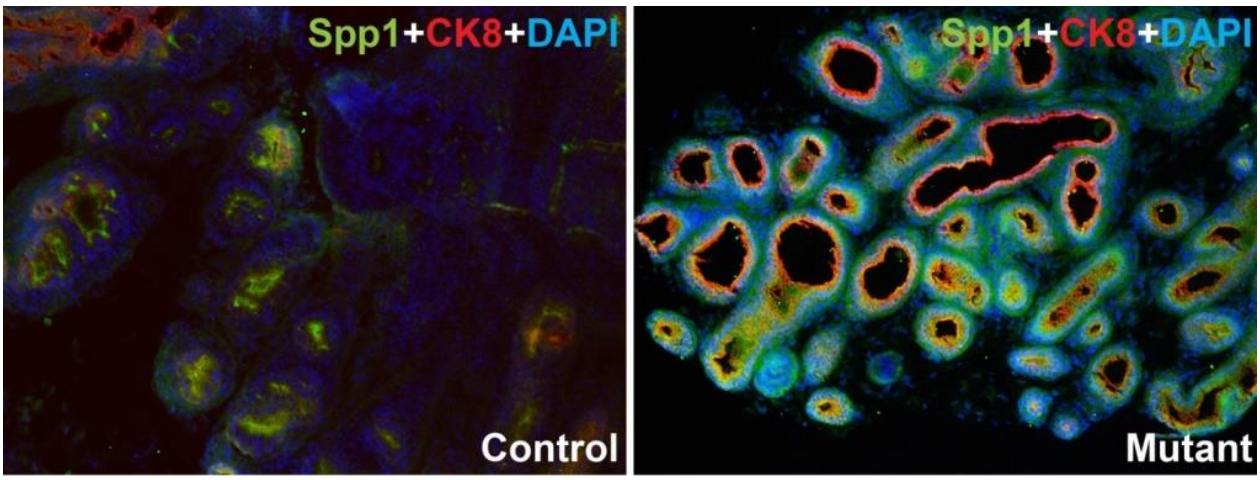

Figure 9: Upregulation of Spp1 in the murine prostate by an inducible transgene.

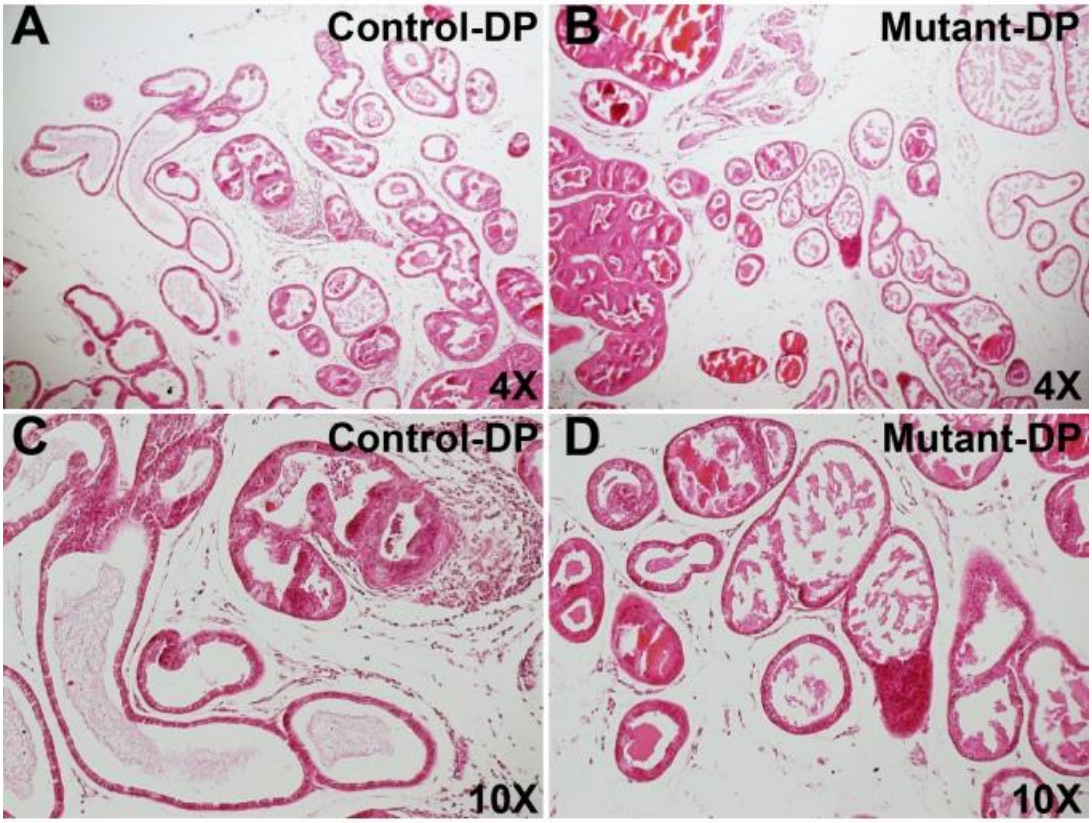

Figure 10: Transgenic upregulation of Spp1 in prostatic epithelium alone did not initiate prostate cancer. *DP: Dorsal prostate.*

The mutants and controls treated with Dox were monitored and analyzed for tumor formation for 12 weeks (**Figure 10**). The prostates from either the control or the mutants did not show any evident of abnormalities. There is no sign of prostatic intraepithelial neoplasia (PIN) or cancer in prostates even after 12 weeks. This is in contrast to NFATc1 mutant mice that develop prostatic adenocarcinoma at 12 weeks of age. These results suggest that SPP1 upregulation by itself is not sufficient to initiate tumorigenesis in the prostate. It may however, play a key role in the progression of prostate cancers as some previous reports suggested.[23]

In addition, we have generated some mice that are *PCre/+, ROSA-rtTA/+, TetO-NFATc1^{Nuc}/+, TetO-Spp1/+*. These mice will have NFATc1 activation and SPP1 upregulation when treated with Dox. While we are still in the process of generating more of the control and mutant groups to perform statistically significant analyses, we will start to treat the initial batch of these mice with Dox and monitor for tumor formation. In parallel, we are generating *PCre/+, ROSA-rtTA/+, TetO-NFATc1^{Nuc}/+, Spp1$^{f/f}$* for NFATc1 activation and germ line SPP1 inactivation to determine if the NFATc1 oncogenic effects will be diminished by the inactivation of SPP1, one of the factors greatly increased in the NFAT-induced prostate cancer and one of the key markers for prostate cancer progression.[23]

Major Task 4: Determine if there is a direct connection between NFATc1 expression and human prostate cancer pathogenesis

4.1: We performed initial study of a potential connection between NFATc1 expression and human prostate cancer progression

Initial analyses using NFATc1 antibody to stain sections from human prostate cancer samples found NFATc1+ cells in the neoplastic epithelium in 18 (~30%) of the adenocarcinoma specimens (n = 57) with Gleason scores ranging from 5–9, but not in the epithelium of non-neoplastic prostates (n = 30). We collected some additional samples adenocarcinoma and found ~30% of total samples had NFATc1 expression.

We have analyzed a small number of human metastatic prostate cancer samples, mainly from lymph nodes (n=11) and bone metastases (n=12). We use immunostaining on sections to look for NFATc1 and E-Cad double positive cells (Figure 11A-B). Almost all of the samples had NFATc1+ cells. We have so far no convincing evidence that any of these NFATc1+ cells are metastatic cancer cells, since most are E-Cad negative. We are continuing our efforts by scanning through more areas of the existing

samples and acquiring more human specimens to increase the sample size and determine if NFATc1 activation is necessary to metastases of prostate cancer.

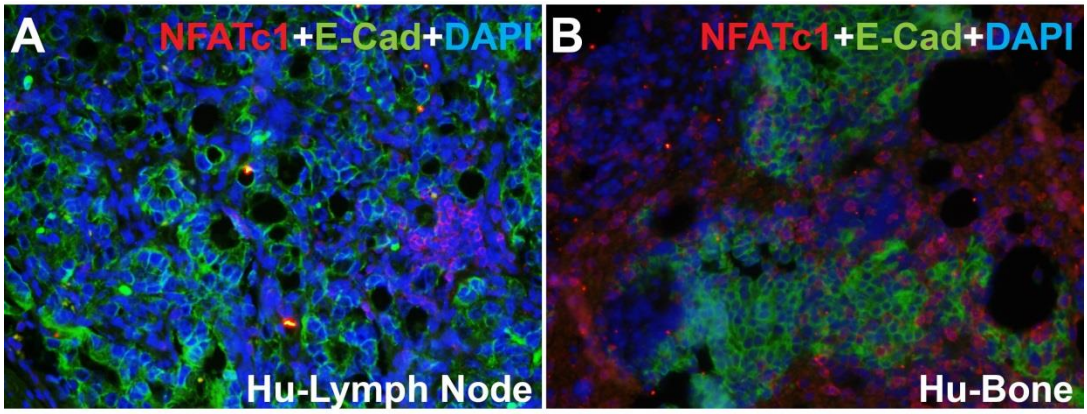

Figure 11: Absence of NFATc1and E-Cad double positive cells in lymph nodes and bone samples with metastatic human prostate cancers.

4.2 Investigate the oncogenic effects of NFAT signaling in human prostate cancer cell lines

We have shown no NFATc1 expression in the prostatic epithelial cell line RWPE1 that is immortalized but not tumorigenic. On the other hand, the metastatic prostate cancer-derived PC3 and DU145 cells have extensive NFATc1 expression and concurrent SPP1 expression.[1] In the next set of experiments, we set out to determine if the modulation of NFAT activity using different inhibitors can alter cellular behavior related to tumorigenesis and cell migration, an important step in cancer metastasis.

The first in vitro assay we did was the clonogenic assay or colony formation assay. We compare the ability of different cell lines to form colonies in the presence or absence of NFAT activation or inhibition (**Figure 12**). Non-transformed prostate epithelial cells RWPE-1 did not express NFATc1 and did not form colonies after 3 weeks. A large percentage of PC-3 cells and LNCap cells have NFATc1 expression. These lines can form colonies I soft agar during the same period of time. The cell line we generated from the NFATc1-induced prostate cancer forms colonies only in the presence of Dox that induces NFATc1 activation, but not in the absence of Dox. These data, especially the results from the cancer cell line we generated, suggest that NFATc1 may promote anchorage-independent growth and transformation.

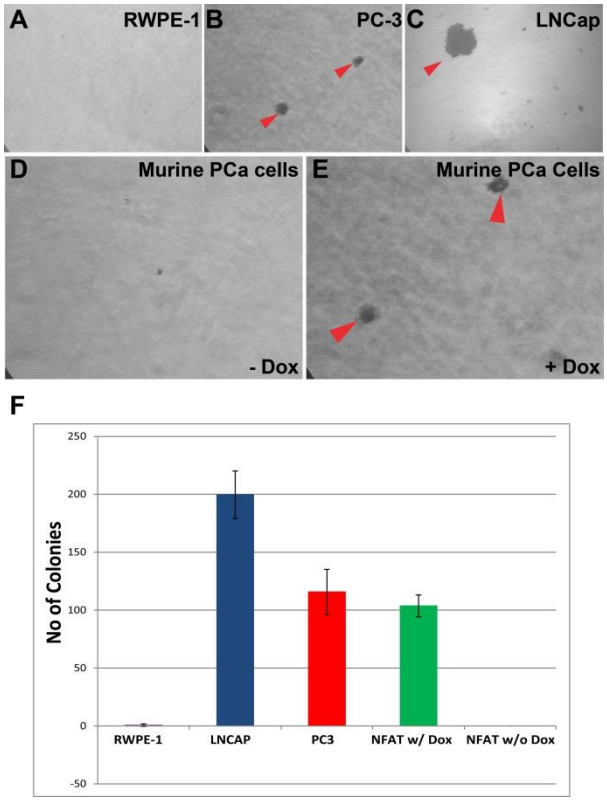

Figure 12: **Colony formation correlates to NFAT activity.** A, Non-transformed prostate epithelial cells RWPE-1 did not form colonies after 3 weeks. B-C, PC-3 cells and LNCap cells form colonies. D-E, the cell line we generated from the NFATc1-induced prostate cancer forms colonies only in the presence but not in the absence of Dox. E, Quantification of the results. P <0.05 when LNCaP, PC3, and "NFAT w/ Dox" were compared to the RWPE-1 data. P <0.05 when "NFAT w/ Dox" and "NFAT w/o Dox" are compared.

To examine if the NFAT activity may affect cell migration, we performed an in vitro scratch wound healing assay and a Boyden chamber transwell invasion assay using the PC3 cells in the presence or absence of NFAT inhibitors. In the wound healing assay, fully confluent PC3 cells in 6 well plates were scratched and allowed to heal in the presence of absence of NFAT inhibitors Cyclosporine A (1uM) and VIVIT (2uM) respectively (**Figure 13**). While mock-treated cells migrate to close the gap significantly 48 hours after the scratches were made, very little has changed in the wells where calcineurin-NFAT inhibitors (Cyclosporine and VIVIT) were present.

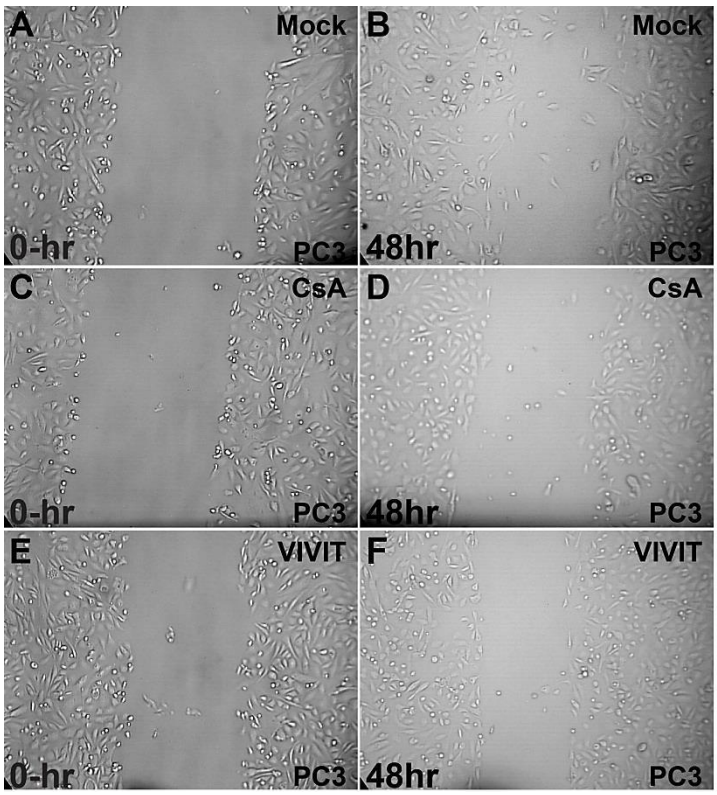

Figure 13: **Inhibition of NFAT prevents/delays scratch wound healing in cultured PC3 cells that express NFATc1.** While mock-treated cells showed significant wound closure after 48 hours (A-B), NFAT inhibition by cyclosporine (C-D) and VIVIT (E-F) negatively impacts the migration of PC3 cells in this wound healing assay.

Similarly, in the Boyden chamber assay, CsA and VIVIT treatment demonstrated marked decreases in the number of PC3 cells migrated across the membrane (Figure 14A-B). On the contrary, more ionomycin-treated cells have migrated when compared to mock-treated cells (Figure 14C-D). Average numbers of cells migrated through the membrane per well (n=3) were 262 ± 31.32 in mock-treated cells. This number is 347 ± 23.24i in onomycin (an Cn-NFAT activator) -treated cells, significantly higher than that of the untreated sample. On the contrary, the numbers of the migrated cells were significantly smaller than the mock-treated control when calcineurin and NFAT inhibitors (CsA: 134 ± 5.56 cells and VIVIT: 130 ± 17.05 cells) were used. These results are consistent with the scratch wound healing results, pointing to a link between NFAT activity and cell migration in these prostate cancer cells.

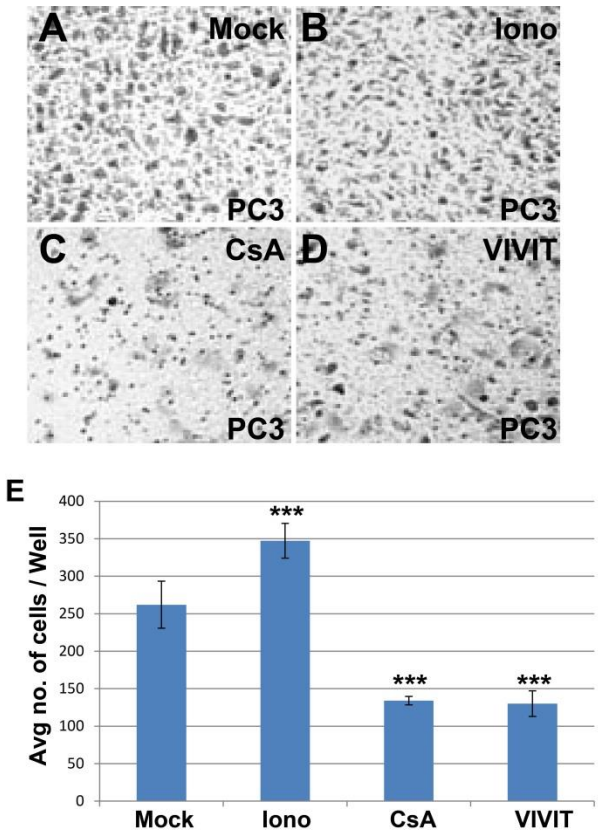

Figure 14: NFAT inhibition affects migration of tumor cells. Inhibition of NFAT in PC3 cells by cyclosporine (C) and VIVIT (D) prevents migration of cells in a Boyden chamber assay whereas ionomycin increases migration when compared mock treated cells (A-B).. *** P ≤0.001 when compared to the mock-transfected cells.

Taken together, results from all the in vitro assays (colony formation, wound healing, and Boyden chamber) appear to indicate that NFAT signaling promotes the transformation, anchorage-independent growth, and migration. Inhibiting this pathway may disrupt and/or reverse some of these processes associated with cancer initiation and progression.

Note: Since this project is still ongoing, future experiments from these studies will provide additional results to accomplish the remaining goals of the award and to further strengthen the results we have.

Key citation: Manda KR, Tripathi P, Hsi AC, Ning J, Ruzinova MB, Liapis H, Bailey M, Zhang H, Maher CA, Humphrey PA, Andriole GL, Ding L, **You Z**, **Chen F**: NFATc1 promotes prostate tumorigenesis and overcomes PTEN loss-induced senescence, Oncogene 2016, 35:3282-3292

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4) Other achievements.

In the past year, besides the above work that is directly related to the funded project, we have performed other studies. Because efforts of the PI (Feng Chen) and the collaborator (Zongbing You) were partially funded by this award, we acknowledged this award in our following publications. In some earlier papers, Dr. You put the award number in the acknowledgement of his papers without mentioning Dr. Chen as the PI, partly because Dr. You is the PI of a subaward based on this award. However, since the last funding period, Dr. You has been putting "(PI: Feng Chen; Co-I: Zongbing You)" in his publications to avoid any misunderstanding.

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Divergent viral presentation among human tumors and adjacent normal tissues
Science Reports 6, 28294.
Status of publication: published; acknowledgement of federal support: Yes.

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Systematic Discovery of Complex Indels in Human Cancers
Nature Medicine 22(1):97-104. PMID: 26657142
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PMID: 27070576
Status of publication: published; acknowledgement of federal support: Yes.

What opportunities for training and professional development has the project provided?
Postdoctoral and other researchers involved in this project were partially supported by this funding. These researchers have gained substantial training in animal disease models and cancer biology as a result of their participation in this research.

How were the results disseminated to communities of interest?
Results have been mainly communicated through scientific publications and meetings at this time.

What do you plan to do during the next reporting period to accomplish the goals?

We will continue the planned research as described in the proposal, including part of subtask 1 of major task 1 and subtask 2 of major task 2 in Aim 1 as well as some experiments in mjor tasks 3 and 4 in Aims 2 and 3. In particular, we will continue to study components of the prostate cancer microenvironment and the interaction of NFAT with key factors in this environment. We will specifically focus on SPP1, Interleukins, Pten, and selected cytokines. We are also generating mice with NFATc1 activation in the prostatic stroma (by using the Tbx18-Cre transgene we made) instead of prostatic epithelium in order to test if changing of the environment is sufficient for prostate cancer development. We will also continue to study murine and human specimens, as well as cultured prostate cancer cell lines, to reveal the involvement of NFAT signaling and related genes in prostate cancer tumorigenesis as described in details in the proposal. Some of the mice necessary for the proposed work will become available in the next period, as will more human specimens. We also believe the analyses of the existing cancer cell lines and the ones we generated from mice carrying NFATc1-induced prostate cancer will provide relatively fast and clean results in the interpretation of the molecular and cellular effects of NFAT signaling in prostate cancer oncogenesis.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

A major challenge for prostate cancer diagnosis/prognosis and treatment is the lack of reliable biomarkers and effective therapeutic targets. In recent years, we have witnessed vigorous debates about the effectiveness and side effects of various prostate cancer screening methods, including the measurement of prostate specific antigen (PSA). It is clear that no single existing marker by itself is sufficient to provide reliable diagnostic/prognostic values and more biomarkers need to be studied to establish an informative matrix to evaluate patients risk and to distinguish aggressive from indolent diseases in prostate cancer. We have shown upregulation of NFATc1 in human prostate cancer specimens and cells. We have also provided the first direct *in situ* evidence in mice that NFATc1 activation induces prostate cancer resembling human prostate cancer. The proposed study is built on these findings and the versatile disease models we generated to further investigate prostate cancer pathogenesis, aiming at revealing the molecular network regulated by NFAT in prostate cancer and the complex interplay between cancer cells and their microenvironment. Successful completion of this study will present NFATc1 and related molecules as potential diagnostic/prognostic markers and novel therapeutic targets in prostate cancer. These results will also enhance our understanding of the regulation of Pten & SPP1, two well-established important factors in human prostate cancer.

What was the impact on other disciplines?

There are more and more evidence that NFATc1 is an oncogene. Studies from pancreatic cancers and many other types of cancers have shown the effects of NFATc1 activation in cancer progression. Our analyses of the cell autonomous and non-cell autonomous functions of NFATc1 in prostate cancer initiation and progression provide a detailed mechanistic explanation of the effects of NFATc1 activation on downstream targets important for cancer development.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

The current funded project is basic research in nature. The primary goal is to better understand the tumorigenic mechanism in prostate cancer with a specific emphasis on NFAT pathway and

microenvironment. Future studies will aim at the translational aspects of the findings to benefit the society directly.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report.

Changes that had a significant impact on expenditures
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

Significant changes in use or care of human subjects
Nothing to Report.

Significant changes in use or care of vertebrate animals.
Nothing to Report.

Significant changes in use of biohazards and/or select agents
Nothing to Report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

Publications, conference papers, and presentations
Report only the major publication(s) resulting from the work under this award.

Journal publications.

The following manuscript/paper was described in last year reported and it is finally in print.

Manda KR, Tripathi P, Hsi AC, Ning J, Ruzinova MB, Liapis H, Bailey M, Zhang H, Maher CA, Humphrey PA, Andriole GL, Ding L, You Z, Chen F: NFATc1 promotes prostate tumorigenesis and overcomes PTEN loss-induced senescence, Oncogene 2016, 35:3282-3292

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.
In the past two years, besides the above work that is directly related to the funded project, we have performed other studies. Because efforts of the PI (Feng Chen) and the collaborator (Zongbing You) were partially funded by this award, we acknowledged this award in our following publications. In some earlier papers, Dr. You put the award number in the acknowledgement of his papers without mentioning Dr. Chen as the PI, partly because Dr. You is the PI of a subaward based on this award. However, since the last funding period, Dr. You has been putting "(PI: Feng Chen; Co-I: Zongbing You)" in his publications to avoid any misunderstanding.

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Nature Medicine 22(1):97-104. PMID: 26657142
Status of publication: published; acknowledgement of federal support: Yes.

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Status of publication: published; acknowledgement of federal support: Yes.

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PMID: 27070576
Status of publication: published; acknowledgement of federal support: Yes.

Invited Lecture:

Nothing to Report.

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products
Cell lines: Murine prostate cancer cells with inducible NFATc1 expression.
Mice: Tbx18-Cre transgenic line, tetO-Spp1 transgenic line

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Feng Chen
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-2307-7954
Nearest person month worked:	2
Contribution to Project:	Dr. Chen was responsible for setting project directions, for administration, supervision of laboratory staff, providing technical help to researchers, organizing analyses, and preparing reports and manuscripts.
Funding Support:	Not Applicable

Name:	Kalyan Manda
Project Role:	Staff Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0001-9666-1759
Nearest person month worked:	8
Contribution to Project:	Perform experiments, analyze data, prepare manuscript.
Funding Support:	Not Applicable
Name:	Piyush Tripathi
Project Role:	Staff Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0001-8337-9316
Nearest person month worked:	5
Contribution to Project:	Perform experiments, analyze data, prepare manuscript.
Funding Support:	Not Applicable
Name:	Zongbing You
Project Role:	Collaborator/Consultant
Researcher Identifier (e.g. ORCID ID):	0000-0001-5048-2229
Nearest person month worked:	0.5
Contribution to Project:	Provide expert advice on the design and execution of the experiments. Help with data interpretation and manuscript preparation.
Funding Support:	Not Applicable

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS
Nothing to Report (not applicable).

9. APPENDICES:
None